

Ovine Fetal Immune Response to Cache Valley Virus Infection

Aline Rodrigues Hoffmann,^{a,*} Piotr Dorniak,^{b,c} Justyna Filant,^{b,c} Kathrin A. Dunlap,^b Fuller W. Bazer,^b

Andres de la Concha-Bermejillo,^{a,d} Christabel Jane Welsh,^{a,e} Patricia Varner,^a John Francis Edwards^{a,*}

Department of Veterinary Pathobiology^a and Center for Biotechnology and Genomics, Department of
Animal Science, Texas A&M University, College Station, TX, USA,^b Center for Reproductive Biology,
Department of Animal Sciences, Washington State University, Pullman, WA, USA,^c Texas Veterinary
Medical Diagnostic Laboratory, College Station, TX, USA,^d and Department of Veterinary Integrative
Biosciences Texas A&M University, College Station, TX, USA^e

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*Corresponding authors: Aline Rodrigues Hoffmann, arodrigues@cvm.tamu.edu, or

John Francis Edwards, jedwards@cvm.tamu.edu.

25 **Abstract**

26 Cache Valley virus (CVV) induced malformations have been previously
 27 reproduced in ovine fetuses. To evaluate the development of the antiviral response by
 28 the early, infected fetus, before development of immunocompetency, ovine fetuses at 35
 29 days of gestation were inoculated in utero with CVV and euthanized at 7, 10, 14, 21 and
 30 28 days post infection. The antiviral immune response in immature fetuses infected with
 31 CVV was evaluated. Gene expression associated with an innate, immune response was
 32 quantified by real-time, quantitative PCR. Up-regulated genes in infected fetuses
 33 included *ISG15*, *Mx1*, *Mx2*, *IL-1*, *IL-6*, *TNF- α* , *TLR-7* and *TLR-8*. The amount of *Mx1*
 34 protein, an interferon stimulated GTPase capable of restricting growth of bunyaviruses,
 35 was elevated in the allantoic and amniotic fluid in infected fetuses. *ISG15* protein
 36 expression was significantly increased in target tissues of infected animals. B
 37 lymphocytes and immunoglobulin-positive cells were detected in lymphoid tissues and
 38 in the meninges of infected animals. These results demonstrated that the infected ovine
 39 fetus is able to initiate an innate and adaptive immune response much earlier than
 40 previously known. That presumably contributes to viral clearance in infected animals.

41

42 **Introduction**

43 Cache Valley virus (CVV) is a mosquito-borne bunyavirus of the family
 44 *Bunyaviridae*, genus *Orthobunyavirus*, Bunyamwera group (40), and endemic in North
 45 America (11). Serologic studies have shown that unlike most members of the family
 46 *Bunyaviridae*, the vectors of CVV also infect larger mammals with virus being isolated

47 from sheep, cattle and horses, and causing reproductive losses in small ruminants (7-9,
48 11-14), similar to the disease caused by Akabane virus (5, 22, 32) and the newly
49 identified Schmallerberg virus occurring in Europe (16). The virus has rarely been
50 associated with meningoencephalitis in humans (4, 41). *In utero* ovine fetal infection
51 causes abortion and fetal malformations, mainly affecting the musculoskeletal (MSS)
52 and central nervous (CNS) systems (7-9, 11, 13, 37).

53 Previous studies describing experimental CVV-induced malformations in ovine
54 fetuses showed that the development of fetal lesions depends on the fetal age when
55 infected. If the virus is inoculated between 28 and 48 days of gestation (dg), fetal death
56 and abortion or MSS and CNS malformations occur. No apparent malformations are
57 observed if the virus is inoculated after 48 dg (7, 11). Virus isolation can only be made
58 from infected fetuses in early development, and viral recovery from tissues of term
59 abortions and malformed lambs is uniformly unsuccessful. The virus is cleared from
60 infected tissues within a few weeks after infection (11, 37) and before the presumed age
61 of fetal immunocompetency, at approximately 70-75 dg (29, 33).

62 The gestation period of the ewe is approximately 147 days. Ovine fetuses develop
63 erythropoiesis, myelopoiesis and megakaryopoiesis in the yolk sac and liver at
64 approximately 17 dg (30). At approximately 20 to 25 dg, lymphocyte production begins
65 in the thymus, and lymphocytes are in the bloodstream at 48-50 dg (30). At 45-50 dg, T
66 and B lymphocytes and cells with surface immunoglobulins are present in the spleen and
67 lymph nodes (27, 28). The lymph nodes become integrated into a lymphatic system
68 around 65 dg (30). Establishing the time when fetuses are able to respond to antigens is

69 difficult. Available data on adaptive immune response of ovine fetuses have been based
70 on serum neutralization assays, which were established for use in mature animals.
71 Additionally, gestation assay points used in experiments are somewhat arbitrary. Ovine
72 fetal antibody response to viral infection at titers greater than 1:2 has been detected after
73 76-78 dg (32). Because the syndesmochorial placenta of ruminants prevents passage of
74 immunoglobulins from the ewe to the fetus (45), antibodies in fetuses and precolostral
75 newborns are those produced by the fetus. For this reason, *in utero* viral infections can
76 be diagnosed in aborted fetuses and stillborn ruminants using serum neutralization tests.
77 Such testing is necessary with CVV because the fetus clears the virus long before the
78 end of gestation (9, 11).

79 Previously, we have demonstrated that fetuses infected with CVV early in
80 gestation (around 35 dg) have low viral antigen and RNA signal in tissues around 56 dg
81 and are able to clear the infection before development of an adaptive immune system at
82 75 dg (37). Similarly, age-based findings have been described in Akabane virus-infected
83 fetuses (5, 29, 32). Because no effective serum neutralization antibody (an adaptive
84 immune response) has been detected in ovine fetuses at the time when CVV is cleared
85 from fetuses (30), it may be that the ovine fetus mounts an innate immune response for
86 early viral clearance. To test this hypothesis, the expression of selected genes associated
87 with the innate immune response was determined in tissues of CVV-infected and non-
88 infected ovine fetuses, and CVV mRNA was quantified in selected tissues of these
89 fetuses to correlate the measured innate response with viral clearance. In addition, fetal
90 Mx protein, an interferon-stimulated GTPase previously associated with antiviral activity

91 against bunyaviruses (15, 23, 25, 38, 44), was quantified in the fetal allantoic and
 92 amniotic fluids. Expression of the *interferon stimulated gene 15 (ISG15)*, that has been
 93 shown to be produced by fetuses in response to viral infection *in utero* (6, 42, 43), was
 94 evaluated in CVV-infected fetal tissues. Finally, distribution of B and T lymphocytes
 95 and immunoglobulin-positive cells was evaluated in infected and non-infected, ovine
 96 fetal tissues in early gestation.

97

98 **Materials and methods**

99 *Virus inoculation and sample harvesting*

100 A group of 15, seronegative, pregnant Rambouillet ewes was housed in BSL2
 101 confinement buildings according to protocols approved by The Institution Animal Care
 102 and Use and the Institutional Biosafety Committee. At 35 dg, ewes were inoculated *in*
 103 *utero* with a 1ml inoculum containing 10^5 50% tissue culture infectious doses (TCID₅₀)
 104 of CVV (infected group) or 1 mL of minimum essential medium (MEM) (mock
 105 infected/control group), as previously described (37). The viral inoculum was derived
 106 from the second passage of an isolate from allantoic membrane from an experimentally
 107 infected fetus (7). At 7, 10, 14, 21 and 28 dpi, three ewes (one mock-infected and two
 108 virus-infected) were humanely euthanized. Selected fetal tissues and their fluids were
 109 harvested and immediately frozen at -80°C or placed in RNA later (Ambion, Life
 110 Technologies, Carlsbad, CA) and frozen at -80°C. The remaining fetal tissues and
 111 placenta were fixed in 10% buffered formalin diluted in deionized water or in
 112 Davidson's AFA (glacial acetic acid, 37% formaldehyde and 95% ethanol) fixative.

113

114 *Real time quantitative polymerase chain reaction (qPCR)*

115 To quantify relative numbers of CVV RNA copies, and abundance of genes of
116 interest, real time qPCR was performed on samples of brain and muscle harvested from
117 CVV-infected or mock-infected ovine fetuses according to a previously published
118 protocol with minor modifications (10). Briefly, RNA was isolated from harvested
119 samples and homogenized in Trizol reagent (Gibco BRL, Bethesda, MD) according to
120 the manufacturer's recommendation. In order to eliminate contamination with genomic
121 DNA, the extracted RNA was treated with the DNase1 amplification reagent (Invitrogen,
122 Carlsbad, CA), and the RNA concentration was quantified by spectrophotometry. RNA
123 quality was determined by denaturing samples in a 1.5% agarose gel electrophoresis
124 stained with ethidium bromide and visualized on a UV transilluminator. The cDNA was
125 synthesized from 500ng of total RNA combined with primer mix containing oligodT
126 primer (0.2 µg/ml), random hexamer primer (300 µg/ml)(Invitrogen), dNTPmix (10mM
127 each) and incubated at 65°C for 5 min. After incubation, SuperScript II reverse
128 transcriptase (Invitrogen) was added to the reaction according to manufacturer's
129 recommendations and reverse transcribed under the following conditions:
130 25°C for 10 min; 42°C for 60 min and 70°C for 5 min. To test for genomic DNA
131 contamination, control reactions were prepared without reverse transcriptase.

132 Specific oligonucleotide primers (Table 1) were generated in Oligo 5 program
133 (Molecular Biology Insights, Inc) for the following genes: M segment of glycoprotein 1
134 of CVV, *interferon-α* (*IFN-α*), *IFN-β*, *TNF-α*, *IL-1*, *IL-6*, *Mx1*, *Mx2*, *ISG15*, *TLR7* and

135 *TLR8*. The real-time qPCR reaction was performed using the ABI prism 7900HT system
 136 (Applied Biosystems, Foster, CA) with power SYBR green PCR Master Mix (Applied
 137 Biosystems). Primer specificity and efficiency ($-3.6 > \text{slope} > -3.1$) were confirmed using a
 138 test amplification run. The data were normalized using cycle threshold (Ct) values for
 139 the ovine *glyceraldehyde 3-phosphate dehydrogenase (GADPH)* gene in each sample.
 140 Semiquantitative analysis was calculated with the $\Delta\Delta\text{CT}$ method, and expressed as
 141 relative fold-changes compared with the lowest value for mock-infected samples. To
 142 calculate relative amounts of viral mRNA, relative fold-changes were compared with the
 143 infected sample with the lowest $\Delta\Delta\text{CT}$.

144

145 *Protein slot blot*

146 The protein slot blot assay was used to determine and compare relative concentrations of
 147 the Mx1 protein in the fetal fluids of infected versus non infected fetuses. The starting
 148 concentration of protein in allantoic and amniotic fluids was determined using the
 149 Bradford Assay as recommended by the manufacturer (Bio-rad Laboratories). According
 150 to the protein concentration, samples were prepared and diluted to 12 μg of protein for
 151 the amniotic fluid and 50 μg of protein for the allantoic fluid in a final volume of 200 μl
 152 of Tris-Buffered saline (TBS). The volume was deposited onto a nitrocellulose
 153 membrane (Bio-Rad Laboratories, Richmond, CA) in a Bio-dot SF micro-filtration
 154 apparatus (Bio-Rad Laboratories) following a modified protocol as previously published
 155 (39). Briefly, nonspecific binding sites were blocked by immersing the membrane in 5%
 156 skim milk in TBS-Tween (TBST) for 1 h at room temperature on an orbital shaker. The

157 membranes were incubated overnight at 4°C in 2.5% milk-TBST containing a rabbit
 158 anti-ovine *Mx1* polyclonal antibody (gift from Dr. Troy Ott, Penn State University) (24)
 159 at a concentration of 1:10000. The membrane was washed three times and then
 160 incubated for 1 h with goat anti-rabbit IgG (1:10000 dilution in 2.5% milk-TBST).
 161 Following incubation with the secondary antibody, the membrane was washed three
 162 times and further incubated for 1 min with Bio-Rad chemiluminescence reagent. The
 163 relative concentration of the *Mx1* protein in triplicate samples was determined
 164 quantitatively in relative light units using the Bio-Rad chemidoc imager and assessed via
 165 use of Quantity One Image analysis software (39).

166

167 *Immunohistochemistry*

168 Immunohistochemistry was performed on 5 µm, deparaffinized sections of
 169 paraformaldehyde-fixed tissues mounted onto positively-charged, silanized slides using
 170 an automated system for immunohistochemistry (DakoCytomation Autostainer®, Dako,
 171 Carpinteria, CA) with minor modifications to the IHC protocol previously described
 172 (37). Antigen retrieval was performed in a decloaker chamber (Biocare Medical,
 173 Concord, CA) with slides soaked in citrate buffer pH 6.0. Sections were incubated with
 174 anti-*ISG15* polyclonal antibody (diluted 1:500) for 30 min (the antibody was a gift from
 175 Dr. Thomas Hansen, Colorado State University) (3), CD3 (diluted 1:400) polyclonal
 176 antibody for 35 min (Dako), anti-CD79a polyclonal antibody (diluted 1:200) for 30 min
 177 (Dako), anti-ovine IgG (diluted 1:500) for 35 min and anti-ovine IgM (diluted 1:2000)
 178 for 30 min (KPL, Inc, Gaithersburg, MA). The primary antibody was followed by

179 incubation with the secondary antibody (MACH 2 anti-rabbit Biocare Medical [CD3,
180 IgG, IgM]; or ImmPress anti-Mouse Ig, Vector Labs [*ISG15*, CD79a]) for 20 min. A
181 chromagen complex, 3,3-diaminobenzidine tetrachloride (DAB, Dako), was used to
182 detect the targeted antigens, and sections were counterstained with hematoxylin. Slides
183 were coverslipped with permount mounting solution. Tissues were evaluated to
184 determine percentage of infected cells in each examined organ and graded as follows:
185 less than 3% of cells positive (mild); between 3-15% of cells positive (moderate); more
186 than 15% of cells positive (marked).

187

188 *Statistical analysis*

189 The quantitative data for mRNA expression and protein levels on fetal fluids were
190 subjected to the Wilcoxon signed rank test to determine differences between infected
191 and non-infected groups. Differences between groups at different dpi were subject to the
192 one pair students t-test. In order to reduce heterogeneity among samples, the data were
193 log-transformed if necessary, and *p*-values below 0.05% were considered statistically
194 significant. The data are presented as means and overall standard errors.

195

196 **Results**

197 *Real-time qPCR*

198 No significant difference in gene expression was observed either between infected
199 animals at each different dpi or between control animals at different dg. Therefore, the
200 data for each gene for each evaluated tissue were pooled into two groups: control and

201 infected animals (Fig. 1). qPCR analysis revealed, that *IL-1*, *IL-6*, *Mx2*, *ISG15*, *TLR7*
 202 and *TLR8* were up-regulated (p -value <0.05) in the brain and skeletal muscle of CVV-
 203 infected fetuses as compared to non-infected fetuses. Only in the brain was *TNF- α*
 204 significantly up-regulated. All examined genes but not *ISG15* and *Mx1*, had higher up-
 205 regulation in the brain as compared to the skeletal muscle (p -value <0.05). Similarly, the
 206 relative number of copies of CVV was significantly higher (p -value <0.05) in the brain
 207 when compared to the skeletal muscle (Fig. 2). Progressively lower means of relative
 208 number of copies of CVV were observed in both brain and skeletal muscle with the
 209 progression of the infection. A statistically significant difference (p -value <0.05) was
 210 observed in the brain of fetuses between 14-28 dpi (CVV in the brain of 10 dpi fetuses
 211 were excluded from analysis due to high variability among those samples), and in the
 212 SKM when comparing 10-14 dpi, 10-21 dpi, and 10-28 dpi.

213

214 *Protein slot blot*

215 A significant (p -value <0.01) increase in the amount of *Mx1* protein in the
 216 allantoic and amniotic fluid occurred in infected fetuses *versus* non-infected fetuses at
 217 each time point examined (Fig. 3). Similar to the quantitative data collected with the real
 218 time qPCR, no effect of day or interaction of day versus infection was detected in
 219 evaluated samples.

220

221 *Immunohistochemistry for ISG15*

222 *ISG15* was detected in several tissues of all infected fetuses, but no *ISG15*
 223 immunolabeling was observed in control fetuses (Fig. 4). In infected fetuses, a strong
 224 signal was observed in numerous cells of the cerebral parenchyma and meninges, and
 225 fewer cells were positive for *ISG15* in the spinal cord. Most fetuses at 7-21dpi had
 226 marked *ISG15* antigen signal in the brain, with moderate signal observed at 28 dpi. The
 227 *ISG15* antigen signal in the spinal cord of infected fetuses varied between mild to
 228 moderate at all test points. Multifocal clusters of cells with positive *ISG15*
 229 immunolabeling were observed in the SKM of infected fetuses through 21 dpi with only
 230 rare cells having a positive signal at 28 dpi. Other tissues with clusters of cells with
 231 positive signal included: heart, smooth muscle of intestine, tongue, fibroblasts in the
 232 subcutaneous tissue, wall of large arteries, cells around small blood vessels, spleen (one
 233 fetus at 21dpi), and rarely in the lungs, tonsils and thymus of earlier infected fetuses.

234

235 *Immunohistochemistry of B and T lymphocytes, IgG and IgM*

236 At 7 dpi/42 dg, scattered CD79a positive cells were observed in the dorsal
 237 abdominal cellular lymphoid aggregates around the abdominal aorta, which correspond
 238 to sites of development of rudimentary renal or mesenteric lymph nodes, and rarely in
 239 the hepatic sinusoids in both control and infected animals. At 10 dpi/45 dg, slightly
 240 increased numbers of CD79a, IgG and IgM-positive cells were observed in the same
 241 locations in infected and control animals (Fig. 5, A-C). Rare IgM-positive cells were
 242 observed in the meninges of one infected animal. At 14 dpi/49 dg, slightly increased
 243 numbers of CD79a, IgG and IgM-positive cells were observed in the sites described

244 above, and at this timepoint, CD79a and IgM-positive cells were in the spleen of one of
 245 the infected animals (Fig. 5, D-F). Surprisingly, another infected animal also had a
 246 marked infiltration of CD79a, IgG and IgM-positive cells in the meninges of the brain
 247 and spinal cord and in rare cells within the parenchyma (Fig. 5, G-I). Increased numbers
 248 of CD79a, IgG and IgM-positive cells were arranged around splenic arterioles in both
 249 infected and control animals at 21 dpi/56 dg and 28 dpi/63 dg, besides also being in the
 250 previously described sites. Numerous IgG and IgM-positive cells were observed in the
 251 meninges of the spinal cord of two infected fetuses at 21 dpi, and rare immunoglobulin-
 252 positive cells were observed in one fetus at 28 dpi. Moderate to marked numbers of CD3
 253 positive cells, evaluated only in sections of brain and transverse sections of the abdomen
 254 including the lumbar spinal cord of control and infected fetuses at 21 and 28 dpi, were
 255 detected in the spleen, renal and mesenteric lymph nodes in all fetuses. Scattered CD3
 256 positive cells were in the meninges of the BRA and SPC of one infected fetus at 14dpi
 257 and in the meninges of the SPC in one fetus at 21 dpi.

258

259 **Discussion**

260 Ovine fetuses are able to mount an immune response associated with CVV
 261 infection earlier in gestation than previously described (22, 29, 32, 33). Although the
 262 fetal immune response was not able to completely clear virus by the end of the study at
 263 63 dg (37), infected ovine fetuses were able to reduce the viral load within the CNS and
 264 MSS. Viral signal was also markedly reduced or absent in the ganglion, retina, kidney
 265 and heart.

266 CVV targets mainly cells within the CNS and MSS, and viral signal was markedly
 267 reduced in the brain and almost completely cleared in the skeletal muscle after 21-28 dpi
 268 in fetuses infected at 35 dg (37). Similarly, a progressive reduction in viral load, as
 269 shown by mean relative amounts of CVV RNA copies was observed in the brain and
 270 SKM in this study, with significant differences observed between most days of infection
 271 in the SKM, and in the brain between 14 and 28 dpi. In addition, in all groups, the
 272 relative number of CVV RNA copies in the brain was significantly higher than in the
 273 skeletal muscle.

274 The *Mx* protein, an interferon stimulated GTPase involved in intracellular
 275 trafficking, membrane remodeling and fusion processes, is capable of restricting growth
 276 of viruses, including influenza virus, measles virus and bunyaviruses (including
 277 orthobunyavirus, phlebovirus and hantavirus genuses) (15, 17-20, 23, 25, 26, 44). In
 278 viral infections, the *Mx* protein acts by interfering with transport of viral components in
 279 infected cells. In a model proposed by Haller and Kochs (2002) (18), *MxA* forms large,
 280 membrane-associated self-assemblies that store monomers of this protein. With
 281 infection, monomers bind to viral target structures, forming new assemblies involving
 282 viral intermediates that lead to mislocalization of viral components and consequent
 283 inhibition of viral replication (18). Human *MxA* has been shown to inhibit replication of
 284 La Crosse virus by binding to the viral nucleocapsid protein, and forming large
 285 copolymers that accumulate in the perinuclear region (25). Previous studies have
 286 demonstrated antiviral activity of bovine *Mx2* in cattle infected with BVDV (21, 42).
 287 Because *Mx* has a demonstrated antiviral effect against bunyaviruses, this study

288 quantified expression of the *Mx* molecule in infected tissues. A significant up-regulation
289 of ovine *Mx1* and *Mx2* was observed in evaluated tissues from infected fetuses compared
290 to controls. Increased secretion of *Mx1* was identified in fetal fluids in infected ovine
291 fetuses. Thus, this protein likely contributes to the clearance of CVV infection.

292 *ISG15* was markedly up-regulated in the brain and in the skeletal muscle and
293 highly expressed in the tissues from infected animals. Similar to the *Mx* protein, *ISG15*
294 is induced by type I interferon, and has been associated with antiviral activity (43). In
295 recent studies, *ISG15* has been shown to conjugate to proteins in a manner similar to
296 ubiquitin (1, 46). Modifications and interference of antiviral signaling pathways
297 involving *ISG15* are mechanisms used by Crimean-Congo hemorrhagic fever virus, a
298 bunyavirus, to evade the innate immune response (1). Up-regulation of numerous ISG,
299 including *ISG15*, and continuous stimulation of the innate antiviral response have been
300 demonstrated in blood and tissues of bovine fetuses and cattle infected with BVDV (21,
301 42).

302 Although *ISG15*, *Mx1* and *Mx2* were abundantly expressed in all infected fetuses,
303 the expression of other selected IFN-stimulated genes was not altered. Previous studies
304 have demonstrated that ovine fetuses within the second and third trimester of gestation
305 are able to produce levels of interferon similar or greater to those produced by adult
306 animals in response to a viral infection (36). Since type I interferon-induced genes were
307 up-regulated, it is presumed that expression of type I interferon genes were also up-
308 regulated at the same or similar times, and that due to the short half-life of type I
309 interferons the timing of up-regulation of the type I interferon(s) was not detected at the

310 time points evaluated. Even though less likely, one must consider the possibility of up-
311 regulation of both ISG15 and ovine Mx by an interferon-independent pathway.

312 CD79a-positive cells (B lymphocytes and plasma cells) were observed in the
313 spleen of CVV-infected fetuses at 49 dg, which is in agreement with previous studies,
314 that identified B lymphocytes in ovine fetal lymph nodes and spleen as early as 47-48 dg
315 (27, 28, 34). Both infected and non-infected fetuses had scattered CD79a-positive, IgM
316 and IgG-positive cells in lymphoid aggregates in the dorsal aspect of the abdominal
317 cavity, sites of development of rudimentary renal and/or mesenteric lymph nodes, as
318 early as 42 and 45 dg, respectively. The identification of CD79a-positive cells in
319 regional lymph nodes earlier than observed in the fetal spleen, supports the previously
320 proposed idea that extrasplenic sites have the potential of producing B lymphocytes (2,
321 34), and potentially explains the fact that splenectomized fetuses are also capable of
322 colonizing Peyer's patches with B lymphocytes (34, 35).

323 A marked infiltration of CD79a, IgM and IgG-positive cells was observed within
324 the meninges with fewer cells within the parenchyma of the brain and spinal cord of
325 infected fetuses at 49 dg. It appears that the ovine fetus not only mounts an innate
326 immune response to a viral infection, but is also able to stimulate an adaptive immune
327 response. Unfortunately, blood samples were not available from the fetuses during these
328 experiments; therefore, fetal neutralizing antibodies could not be assayed. In ovine
329 fetuses infected with Akabane virus, the earliest day of gestation where IgM and IgG-
330 positive cells were observed in tissues was 59-60 days gestation. Titers >1:4 were
331 detected only after 100 dg (22, 31). Two other studies detected Akabane virus

332 neutralizing antibodies after 76-78 dg, with titers ranging from 8-64 in one of these
333 studies (29, 32).

334 CVV is a viral pathogen with a tropism for the ovine fetal CNS, skeletal muscle
335 and fetal membranes. The study of early infection demonstrated that a tropism correlated
336 well with the CNS and musculoskeletal malformations observed in spontaneous CVV
337 disease. With CVV, the development of arthrogryposis probably has a multifactorial
338 pathogenesis involving effects on developing neurons, myocytes, and fetal membranes.
339 CVV is the only viral infection shown to cause oligohydramnios. The virus is partially
340 cleared from most fetal tissues by approximately 65 dg (28dpi), before the presently
341 accepted onset of ovine immunocompetence.

342 The ovine fetus is able to mount an immune response associated with CVV
343 infection earlier in gestation by up-regulation of genes that participate in the innate
344 immune response. The infected fetus up-regulates *Mx* genes, which have been shown to
345 restrict growth of bunyaviruses. In addition, cells of the adaptive immune response,
346 CD79a-positive, IgM and IgG-positive cells, are found in large numbers in infected
347 tissues. Whether the immunoglobulin in these cells is actually CVV specific is still
348 unclear. Further studies should be conducted for better understanding of the immune
349 system development in the ovine fetuses exposed to CVV and other viral infections.

350

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359

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494
495

496 Table 1. Primer sequences used for RT-qPCR.

Gene Symbol	Accession	Primer Sequences	
		Forward	Reverse
<i>CVV</i>	AF082576.1	CAC CAG CGA AAT CCC AAT CAC CA	CTC CAG ACA TAG CAC CCA CCA
<i>TNF-α</i>	X55152.1	CGG CGT GGA GCT GAA AGA CAA	CTG CGA GTA GAT GAG GTA AA
<i>IL-1</i>	NM_001009465.2	AGT GAT GGC TTG CTA CAG T	CCG AGG TCC AGG TGT T
<i>IL-6</i>	X68723.1	GAG GGA AAT CAG GAA ACT GT	CTC GTT TGA GGA CTG CAT CT
<i>TLR7</i>	HQ529279.1	ACT CCT TGG GGC TAG ATG GT	GCT GGA GAG ATG CCT GCT AT
<i>TLR8</i>	FJ905847.1	TCC ACA TCC CAG ACT TTC TA	GTT CTT GTC CTC ACT CTC TT
<i>Mx1</i>	JN377734.1	GTA CGA GCC GAG TTC TCC AA	ATG TCC ACA GCA GGC TCT TC
<i>Mx2</i>	NM_001078652.1	CAT CCA TAA ATC GCT CCC CTT GT	GCT CCT CTG TCG CCC TCT GGT
<i>ISG15</i>	FJ844480.1	TGA CGG TGA AGA TGC TAG GG	ACT GCT TCA GCT CGG ATA CC
<i>IFN-α</i>	X55152.1	ACC CAG CAC ACC TTC CAG CTC TT	CCT CGC AGC CCC TCC TC
<i>IFN-β</i>	EU276065.1	TGG TTC TCC TGC TGT GTT TCT C	CGT TGT TGG AAT CGA AGC AA

497

498 Figure legends:

499 Figure. 1. Relative levels of genes expression in brain and skeletal muscle of control and Cache
500 Valley virus infected animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

501

502 Figure 2. Relative number of Cache Valley virus RNA copies in main target tissues, brain (BRA)
503 and skeletal muscle (SKM) after 10, 14, 21 and 28 dpi. * $p < 0.05$, ** $p < 0.01$.

504

505 Figure 3. Relative amounts of ovine *Mx1* protein in allantoic and amniotic fluid in control and
506 infected animals. The presented values (y axis) are the relative light units using the Bio-Rad
507 chemidoc imager and assessed via use of Quantity One Image analysis software. The starting
508 protein concentration for the samples from the allantoic fluid was 50 μg and for the amniotic
509 fluid was 12 μg . The data was log transformed for normalization. The infected fetuses had
510 relatively higher amounts of *Mx1* protein in the allantoic and amniotic fluid when compared to
511 the control fetuses. $p < 0.005$

512

513 Figure 4. Immunohistochemistry for *ISG15* in brain and skeletal muscle. A rabbit polyclonal
514 antibody against ovine *ISG15* protein was stained with DAB chromagen (brown) on the brain
515 (A,B,C) and skeletal muscle (D,E,F) from 45 dg control fetuses (A,D) and from infected fetuses
516 at 14 dpi (45 dg) (B,E) and 28 dpi (63 dg) (C,F). Numerous cells in the brain and skeletal muscle
517 of infected fetuses are positive for *ISG15*. No positive cells were observed in tissues of control
518 fetuses.

519

520 Figure 5. Immunohistochemistry for B cells, plasma cells and ovine IgG and IgM in lymph node,
 521 spleen and brain. A polyclonal antibody against B cells and plasma cells (CD79a), ovine IgG and
 522 ovine IgM was stained with DAB chromagen (brown). CD79a (A), IgG (B) and IgM (C) positive
 523 cells were observed in rudimentary lymph nodes at 42-45dg in control fetuses. CD79a (D), IgG
 524 (E) and IgM (F) positive cells were observed in the spleen of infected fetuses. A remarkable
 525 number of CD79a (G), IgG (H) and IgM (I) positive cells were seen in the leptomeninges of an
 526 infected fetus at 14 dpi.









